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Analysis by detached leaf assay of components of partial resistance of faba bean (*Vicia faba* L.) to chocolate spot caused by *Botrytis fabae* Sard.

Aïcha Bouhassan[1], Mohammed Sadiki[2], Bernard Tivoli[3] and Najat El Khiati[1]

[1] Faculté des Sciences Ain Chock, Université Hassan II, Laboratoire de Biologie et Physiologie Végétale, B.P. 5366, Maârif, Casablanca, Morocco
[2] Institut Agronomique et Vétérinaire Hassan II, Laboratoire de génétique des légumineuses, Département d'Agronomie et d'Amélioration des plantes, B.P. 6202, Rabat, Morocco
[3] Institut National de la Recherche Agronomique de Rennes, Station de Pathologie Végétale, Domaine de la Motte, B.P. 29, F-35663, Le Rheu Cedex, France

Summary. Five faba bean (*Vicia faba* L.) genotypes with different levels of field susceptibility to chocolate spot caused by *Botrytis fabae*, were analysed for the components of partial resistance in a laboratory assay on living detached leaves. The incubation period (IP), the number of spots (NP), the lesion diameter (LD), the latency period (LP) and the number of spores/leaflet (NS), were determined and statistically analysed. Parameters LD, LP and NS were involved in characterising partial resistance, but IP did not appear to play a role. NS did not become a significant factor until 20 h after inoculation. Genotype FRY167 was most resistant with the lowest LD and NS values and the longest LP. In contrast, FRY30 had the highest level of susceptibility, with the highest LD and NS and the shortest LP. However, the resistant control BPL710 showed a slight deviation in that it expressed a long LP which was not significantly different from the values of the susceptible control Aguadulce. The absence of a correlation between LP and NS in BPL710 opens a discussion on the genetic control of its resistance.

Key words: AUDPC, incubation period, lesion diameter, latency period, disease scores.

Introduction

Chocolate spot caused by *Botrytis fabae* is the most destructive faba bean disease in the world (Sundheim, 1973; Tivoli *et al.*, 1988) and is particularly threatening in the Mediterranean basin (Hanounik, 1979; Maatougui *et al.*, 1994; Mabsoute and Saadaoui, 1996). In Morocco, yield losses exceed 60%, and, under favourable conditions, may reach 100% (Mabsoute and Saadaoui, 1996; Sadiki *et al.*, 2000). Chemical control is possible but is environmentally unfriendly and expensive. An effective control strategy should therefore include a host resistance component.

In faba bean, germplasm collections are screened under field and laboratory conditions to identify sources of resistance to chocolate spot (Jellis *et al.*, 1982; Hanounik and Robertson, 1988; Tivoli *et al.*, 1988, 1992; Santorelli *et al.*, 1992). Known sources of resistance are still very few, although some resistant genotypes have been identified. The resistance expressed by these genotypes has not been thoroughly analysed to distinguish

Corresponding author: A. Bouhassan
Fax: +212 2 23 06 74
E-mail: bouhassan@hotmail.com

between total resistance, which is qualitative or race-specific, and partial resistance, which is quantitative, or non-race specific (Van Der Plank, 1963). Partial resistance remains the more desirable alternative because of its stability and durability over time and across space (Johnson, 1979). Partial resistance is expressed as a slower development of the pathogen and its sporulation process (Parlevliet, 1979). In most studies, the main selection criterion for resistance has been the evolution of disease symptoms. To our knowledge, no detailed study on the components of partial resistance to faba bean chocolate spot has yet been published.

The main epidemiological components of partial resistance are infection efficiency, extent of symptoms, and latent period, defined as the time necessary the formation of the first spores and the rate and duration of sporulation (Van Der Plank, 1963; Parlevliet, 1979). An analysis of the contribution of each of these components may show its value in selecting for partial resistance, as in the case of the latent period of rust in wheat (Broers, 1997). Similarly, components such as infection efficiency and sporulation rate are used to select rice for resistance to *Pyricularia oryzae* (Yeh and Bonman, 1986). In addition, the combination of these components can enhance the quantitative resistance of some cultivars (Parlevliet, 1979). The present study analysed partial resistance to *B. fabae* in faba-bean resistant and susceptible genotypes. The objectives were to analyse the epidemiological components of partial resistance, to ascertain the contribution of individual components to the expression of this resistance, and to evaluate a rating scale that can be used in selection for resistance.

Materials and methods

Plant production and inoculation

All experiments were carried out on 5 genotypes or lines selected from the DBFRYMED collection (Sadiki *et al.*, 2001). FRY167, FRY7 were resistant and FRY30 susceptible genotypes in the experimental field of the Société de Gestion des Terres Agricoles (SOGETA) in Ain Dick, Rabat, Morocco in 1998 and 1999 (Bouhassan *et al.*, 2000). BPL710, a resistant line from the ICARDA collection (Jellis *et al.*, 1982), and the genotype Aguadulce were used as resistant and susceptible controls respectively. Seeds were planted on March 14, 2000 in 12-cm-diameter pots filled with a mixture of arable soil, peat and sand 3:1:1 (v:v:v). Five seeds were planted per pot. The plants were exposed to temperature conditions varying from 20 to 24°C, with a photoperiod of 13 h.

Two hours prior to inoculation, leaves were harvested from 3 randomly selected plants per line. Leaves were taken from the last but one foliar node. In the laboratory, one leaflet was removed from each of the detached leaves and these leaflets were immediately placed in Petri dishes on 2 layers of filter paper, previously sterilised and wetted with distilled water. Small humid pieces of cotton (about 4-mm diameter) were put at the end of the leaflet petioles to maintain cells at maximum turgescence. Leaflets were randomly distributed in the Petri dishes.

Inoculum was prepared from a single *B. fabae* strain (BFIAV99-1) previously isolated from leaves collected in the Rabat region. To induce spore formation, the fungal culture was transferred to a faba bean leaf extract medium as described by Leach and Moore (1966). After 10 days of incubation at 20–22°C, the surface of each colony was covered with 5–10 ml of sterile water. The spores were dislodged from the surface of the agar by passing a curved Pasteur pipette gently over it. The water suspension thus obtained was filtered through two layers of sterile gauze and diluted with tap water. The spore concentration was adjusted to 3×10^6 spores ml^{-1} with a Malassez haemacytometer slide (OSI, LAB'85, Paris, France).

Leaflets were inoculated between midnight and 1 am by dropping 20 μl of the prepared suspension on each leaflet with a micropipette. The Petri dishes with the material inoculated were immediately placed in a growth chamber at 18±2°C with a 14-hour day.

Components of partial resistance

Each leaf was examined under a stereomicroscope to determine the incubation period (IP), defined as the number of hours necessary for first symptom appearance. The number of spots (NP) at the inoculation site was counted 8, 11, 14, 17, 20, 24 and 30 h following inoculation.

Disease progress was assessed daily starting 48 h after inoculation by measuring the lesion diameter (LD).

Daily examination of the lesions also made it possible to determine the latent period (LP) (Parlevliet, 1979).

The two last procedures were carried out under laminar airflow (sterile conditions).

Eleven days after inoculation 3 leaflets of each genotype were washed separately in a fixed volume of distilled water to collect the spores. The number of spores per ml of each suspension was counted under the microscope using a Malassez haemocytometer. Average spore production (NS) expressed as the number of spores/leaflet was then determined.

During the 9 days of incubation, disease intensity was scored using the following 9-point rating scale: 1, beginning of spotting; 2–4, clearly individualised spots increasing in number and size; 5, brown spots, some mm in diam., occupying approximately 10% of the surface of the leaflet; 6, spots of larger size, beginning of sporulation; 7–8, spots occupying 1/3 to 2/3 of leaflet area; 9, spots covering more than 90% of leaflet area, with numerous spores. The goal was to compare results obtained by rapid evaluation without reference to sporulation with results obtained by a detailed and precise study of the components and closely linked to different stages of the fungus life cycle.

Statistical analysis

The area under the disease progress curve (AUDPC), was computed for number of spots, size of spots and disease scores according to the following formula (Shaner and Finney, 1977):

$$\text{AUDPC} = \sum_{i=1}^{n} {}^{1}/_{2}\left[(y_{i+1} + y_i)\,(x_{i+1} - x_i)\right]$$

where xi = time (days or h); yi = disease severity at the day i (or time i); and n = total number of symptom observations.

Analysis of variance and the least significant difference test (LSD) were performed on the data for each factor obtained for the 5 genotypes using the SAS statistical software package (SAS Institute, Cary, NC, USA).

Results

Components of partial resistance

Incubation period

The IP varied from 9 h (FRY30) to 13.3 h (FRY167), but the 5 genotypes were not significantly different from eachoter (P=0.23) (Table 1). The IP for the two controls (resistant and susceptible) were also not significantly different; they were 11.2 h for the resistant control and 11.5 h for the susceptible control.

Number of spots

In the hours immediately following inoculation, the first symptoms of the disease appeared at the inoculation site as small brown dots increasing rapidly in number and generally fusing in 24–30 h to form a spot several mm in diam.

The number of these dots showed significant differences between various genotypes at 8, 11 and 14 h after inoculation (Table 1). At 8 h, FRY167 had the fewest spots (0.4) and FRY30 the most (6.9). After 14 h, the genotypes eventually fell into two significantly different groups according to AUDPC values: the first contained the genotype FRY30 and the susceptible control Aguadulce, the second the genotypes FRY167 and FRY7, and the resistant control BPL710. Between 17 and 30 h, the differences among these genotypes were not however significant (Table 1).

Table 1. Incubation period (hours), average number and AUDPC of spots per leaflet (8 to 30 h after inoculation), of five faba bean genotypes inoculated with a single virulent isolate of *Botrytis fabae*.

Genotype	Incubation period (h)	Spots per leaflet (No.)								
		No. of hours after inoculation								AUDPC
		8.0	11	14	17	20	24	30	8–30	8–17
FRY167	13.3 a	0.4 c	3.3 c	10.8 c	17.6 b	29.3 a	29.3 a	64.7 a	624.8 a	69.4 b
FRY7	11.2 ab	1.6 bc	5.7 bc	13.9 bc	21.6 ab	37.1 a	47.4 a	70.1 a	702.9 a	93.6 b
BPL710	11.5 ab	2.0 bc	4.9 c	9.0 c	20.7 ab	36.7 a	46.7 a	69.1 a	675.8 a	75.6 b
Aguadulce	11.3 ab	5.6 ab	14.1 ab	25.1 ab	39.8 a	57.3 a	61.1 a	73.3 a	971.3 a	185.5 a
FRY30	9.0 b	6.9 a	17.1 a	30.1 a	39.1 a	57.4 a	65.5 a	80.3 a	1038.4 a	210.7 a
$LSD_{0.05}$	3.6	4.8	8.8	13.5	20.8	29	34	37.6	490.8	101.3

Values in columns followed by different letters indicate significant differences (P<0.05) according to the LSD test.

Table 2. Average lesion diameter and AUDPC of lesions (mm) in five faba bean genotypes inoculated with a single virulent isolate of *Botrytis fabae* (measured from day 2 to day 9 after inoculation).

Genotype	Average lesions diameter (mm)								
	No. of days after inoculation								AUDPC
	2	3	4	5	6	7	8	9	Lesions
FRY167	2.8 b	5.8 c	11.3 a	11.9 b	12.8 b	14.7 b	15.4 b	17.2 b	83.7 c
FRY7	3.9 ab	6.9 bc	10.5 a	12.5 b	14.7 b	16.5 b	17.9 b	19.5 b	93.3 c
BPL710	4.6 ab	7.7 abc	12.3 a	14.7 ab	17.3 b	18.4 b	20.1 b	23.3 b	107.5 bc
Aguadulce	4.8 a	10.1 ab	15.4 a	20.5 a	25.9 a	27.7 a	29.3 a	33.3 a	151.6 ab
FRY30	5.2 a	10.9 a	15.9 a	21.9 a	25.9 a	29.4 a	32.1 a	35.0 a	160.5 a
$LSD_{0.05}$	1.4	3.9	6.4	7.4	8.1	8.2	8.7	8.9	48.1

Values in columns followed by different letters indicate significant differences ($P<0.05$) according to the LSD test.

Analysis of the rate of spread of the spots revealed that spread was slower in the resistant than in the susceptible genotypes: after 14 h, it was 0.8 dots h^{-1} in FRY167 and 2.1 dots h^{-1} in FRY30.

Diameter of lesions

Difference in LD between resistant and susceptible genotypes became significant only from day 6 after incubation. The LD then defined FRY30 and Aguadulce as susceptible and FRY167, FRY7 and BPL710 as resistant (Table 2). Comparison with AUDPC revealed a highly significant genotypic effect ($P=0.004$). Genotypes FRY167 and FRY7 were more resistant than the resistant control BPL710, and conversely FRY30 was more susceptible than the susceptible control Aguadulce. However, the two controls BPL710 and Aguadulce were significantly different only from day 6 of incubation (Table 2). Nevertheless, the differences between their AUDPC values were not significant, due to the similarity of their LD for the first 5 days after infection. During the experiment, the average rate of spot spread varied from nearly 2 mm day^{-1} in FRY167 to 4 mm day^{-1} in FRY30.

Latent period

The LP differed significantly between genotypes (Table 3), from 4.7 days to 7 days. The two susceptible genotypes had a short LP. Of the three resistant genotypes, FRY167 and FRY7 had a long LP; however, that of BPL710 was short and was not significantly different from the susceptible genotypes. The LP of the resistant genotypes FRY167 and FRY7 was two days longer than that of the susceptible genotypes and the resistant control BPL710.

Number of spores per leaflet

The mean NS produced by each genotype after 11 days of contact with the pathogen is shown in Table 3. Differences between genotypes were highly significant, FRY167 having the lowest spore count (13×10^5), and FRY30 the greatest (3.43×10^5). This component again confirmed the separation of the resistant and susceptible groups, and it also distinguished between the two controls, which differed highly significantly in their NS, BPL710 clustering with the resistant genotypes FRY167 and FRY7, and Aguadulce with the susceptible genotype FRY30.

Overall disease score

Table 4 shows that differences between genotypes in the visual disease score became significant only

Table 3. Latent period (days) and sporulation capacity of five faba bean genotypes inoculated with a virulent isolate of *Botrytis fabae*.

Genotype	Latent period (days)	Number of spores per leaflet (10^5)
FRY167	7.0 a	1.3 b
FRY7	6.6 a	1.7 b
BPL710	5.0 b	1.9 b
Aguadulce	5.0 b	3.0 a
FRY30	4.7 b	3.4 a
$LSD_{0.05}$	1.5	0.8

Values in columns followed by different letters are significantly different ($P<0.05$) according to the LSD test.

Table 4. Mean disease score and AUDPC of disease scores for five faba bean genotypes inoculated with a single virulent isolate of *Botrytis fabae* from day 1 to day 9 after inoculation.

Genotype	Mean disease score 1–9 days after inoculation									AUDPC
	1	2	3	4	5	6	7	8	9	
FRY167	0.8 b	2.3 a	2.7 b	3.7 c	3.9 c	4.0 d	4.2 d	4.5 c	4.7 c	28.2 b
FRY7	1.1 b	2.8 a	3.5 ab	4.1 bc	4.4 c	5.1 cd	5.1 cd	5.4 c	5.6 bc	33.7 b
BPL710	1.3 b	3.3 a	3.9 ab	4.9 abc	4.9 bc	5.5 bc	5.7 bc	5.9 bc	6.1 b	07.8 ab
Aguadulce	2.5 a	3.5 a	4.1 ab	5.3 ab	6.0 ab	6.4 ab	6.9 ab	7.2 ab	7.6 a	44.4 a
FRY30	2.7 a	3.7 a	4.7 a	5.6 a	6.5 a	6.9 a	7.2 a	7.5 a	7.7 a	47.3 a
$L.S.D_{0.05}$	1.1	1.4	1.5	1.4	1.4	1.3	1.3	1.4	1.3	10.5

Values in columns followed by different letters are significantly different (P<0.05) according to the LSD test.

on day 5 after inoculation, except for the extreme genotypes FRY167 and FRY30 which were clearly and significantly separated during the entire infection period. However, the two controls BPL710 and Aguadulce did not differ significantly. The AUDPC values showed highly significant differences between genotypes (P=0.003) separating resistant and susceptible genotypes, but they did not separate the two controls, whose differences did not become significant. The ranking test of the genotypes based on the 9-point disease score showed a significant separation of the two groups already shown to be different on the basis of the other components. The separation of the groups by this parameter was significant from day 5 following inoculation. Additionally, AUDPC differences between genotypes were highly significant (Table 4).

Correlation between parameters

Table 5 shows the Pearson correlation coefficients between the components of partial resistance. The IP was not correlated with any other component. The number of spots was highly correlated with LD (r=0.98; P=0.004) and with NS (r=0.98; P=0.001) (Fig. 1a, b). The NS was highly correlated with LD (Fig. 1c). However, the LP was not significantly correlated with any other component (Table 5). This was mainly due to the behaviour of BPL710 (Fig. 1d, e). Indeed, when this line was excluded from the analysis, there were highly significant negative correlation coefficients between LP and LD (r=0.99), and between LP and NS (r=-1).

Discussion

The present study analysed *in vitro* a number of epidemiological parameters that characterise partial resistance in certain pathosystems (Roumen and De Boef, 1993; Broers, 1997), but that have not previously been studied on the faba bean-*B. fabae* system. These epidemiological parameters were analysed to characterise the partial resistance of 5 faba bean lines to *B. fabae*.

In the hours immediately following contact between the pathogen and the leaves, the number of spots was an important parameter for evaluating resistance, and could be used instead of incubation period, which was not significant. However, this criterion was applied only for the first 11 h after inoculation. Indeed, *B. fabae* colonises faba bean tissue soon after penetration. Differences in number of spots

Table 5. Pearson correlation coefficients (P<0.05) between the components of partial resistance analysed on five faba bean genotypes inoculated with a single virulent isolate of *Botrytis fabae*.

Components[a]		Correlation coefficient
IP	NP	0.79
IP	LD	0.78
IP	LP	0.72
IP	NS	0.84
NP	LD	0.97**
NP	LP	0.73
NP	NS	0.98**
LP	LD	0.84
LP	NS	0.82
NS	LD	0.99**

[a] IP, incubation period; NP, number of spots; LD, lesion diameter; LP, latent period; NS, number of spores per leaflet.
** Significant at 0.01 probability level.

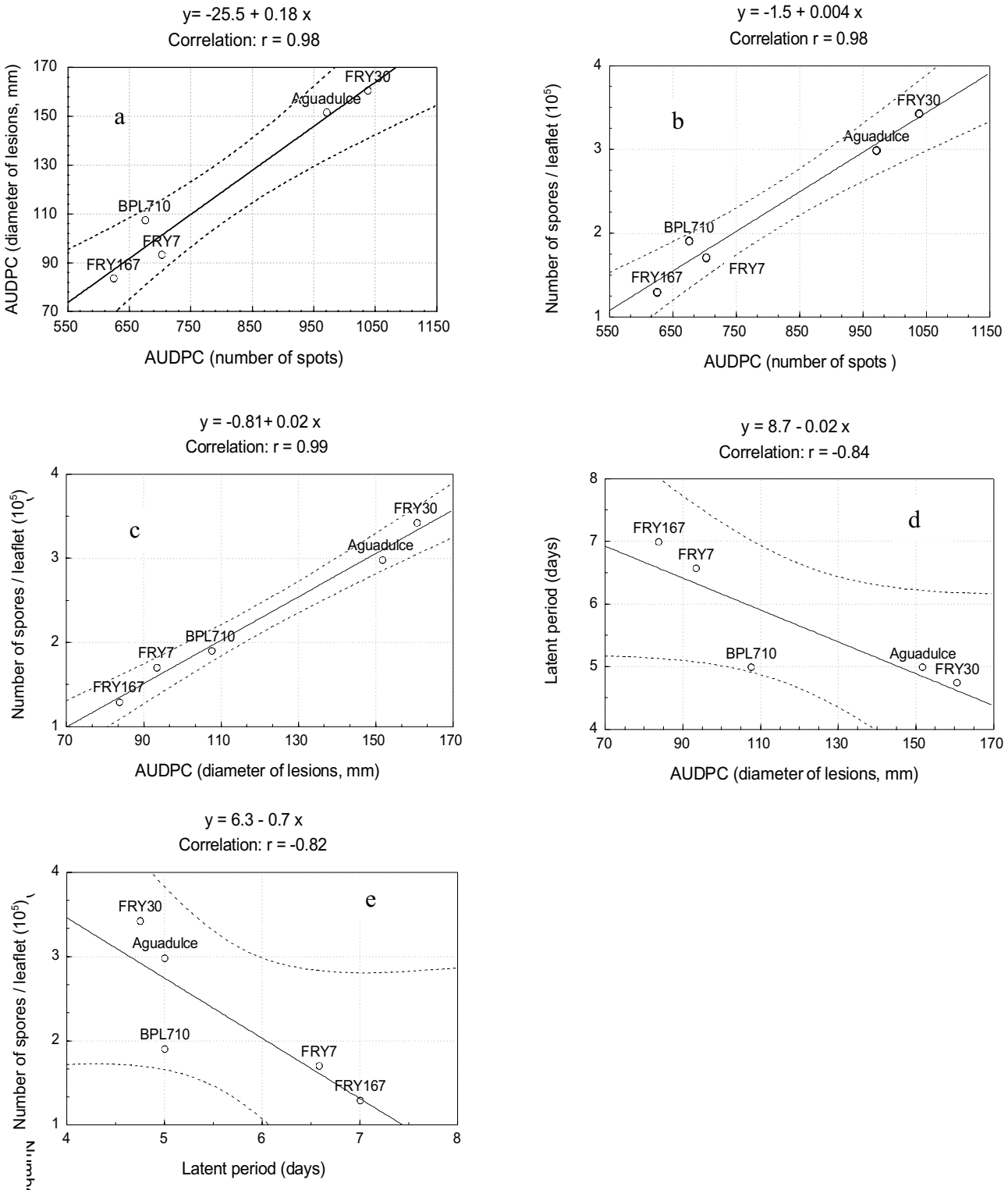

Fig. 1: Relationship between means of components of partial resistance (1a–1e) analysed on detached leaves of five faba bean genotypes inoculated with a single virulent isolate of *Botrytis fabae* (confidence belts for the regression at 95%).

between genotypes in the hours immediately following contact with the pathogen were probably due to early resistance to fungal penetration by the host plant. Such resistance may take the form of a reduction in the number of appressoria, in the number or size of germinating tubes, or even in the mass of hyphae, as described on oat varieties infected with *Erysiphe graminis* (Carver and Adaigbe, 1990).

The LD was an important parameter since the difference between resistant and susceptible genotypes did not manifest itself until after day 6 of incubation, suggesting that the other defence mechanisms of the leaves arose late. Indeed, pathogen multiplication was slower in the resistant genotypes, limiting leaf colonisation.

By means of this parameter, which is a measure of infection efficiency, genotypes FRY167, FRY7 and BPL710 expressed their level of resistance. This parameter is also reported as a good component of partial resistance in other pathosystems (Viljanen-Rollinson *et al.*, 1998).

LP and NS were important parameters of partial resistance to chocolate spot in FRY167 and FRY7. These genotypes had a longer LP and a lower NS than FRY30 and Aguadulce.

It is evident that the resistance clearly shown by FRY167, preventing the appearance of the first spots and lowering the rate of disease spread, prolonged its effects by delaying the LP and reducing the NS. In the case of BPL710 however, LP did not seem to be a convenient parameter to characterise its resistance. The LP of this line (5 days) was similar to that of the 2 susceptible genotypes, and it was significantly different from that of FRY167 and FRY7 (7 and 6.6 days respectively). Several authors have reported considerable differences in LP between cultivars (Broers, 1997). However, Roumen and De Boef (1993) found that LP was not an important component of partial resistance to leaf blast in rice. They suggested that differences in LP between cultivars might not be very clear when the isolate was virulent. This may well be the case of BPL710 used for inoculation against the *B. fabae* isolate in the present experiments.

The lack of any significant correlation between LP and NS may suggest different genetic controls. Nevertheless, the linear regression made it possible to detect the deviation of the genotype BPL710. When this genotype was dropped from the analysis the correlation was greatly increased. Such behaviour may be explained by the fact that BPL710 differed from other genotypes in how it controlled resistance. In that case, it is possible that certain components of partial resistance of this genotype were dependent upon minor genes with possible high levels of unicellular hyper-susceptibility. This phenomenon was observed in a barley variety expressing partial resistance to *Erysiphe graminis* (Asher and Thomas, 1983).

In sum it seemed clear that the components LD, LP and NS were generally correlated with each other and acted in association to define partial resistance of faba bean to *B. fabae*. Therefore, the use of one of these components should be sufficient to determine the level of partial resistance in a genotype. The total spore count nevertheless represented the best parameter as it was significantly correlated with all the other components and made it possible to separate genotypes according to their degree of resistance. Several authors have demonstrated the value of this parameter as a measure of the partial resistance of plants (Vallavieille-Pope *et al.*, 2000).

In our study FRY167 was the most resistant genotype, being distinguished from the rest by the lowest rate of the disease spread in the early stage of incubation, the longest LP and the lowest NS. It was followed in terms of resistance by FRY7. FRY30 was highly susceptible, with the largest LD, the shortest LP and the greatest NS. It was followed by the susceptible control Aguadulce. These different resistance levels were easily detectable with the disease intensity score, whether on detached leaves or on entire plants inoculated in the field (Bouhassan *et al.*, 2000). The disease score included the different phases of the fungus cycle and perfectly distinguished different levels of susceptibility and resistance of the plant material. This scoring method, avoiding a long counting process and complex measurements thus presents important practical advantage for breeding programs.

The miniaturised test on detached leaves maintained alive is very appropriate for analysing the components of partial resistance to *B. fabae* in faba bean. Being simple it allows such analysis to be carried out without the biasing effects of environmental factors, including the action of other types of micro-organisms that are not easily controlled in field experiments. The detached leaf assay was also successfully tested for partial resistance on peas infected with powdery mildew (Warkentin *et*

al., 1995) and for resistance of *Amelanchier alnifolia* Nutt. to *Entosporium mespili* DC. (Ronald *et al*., 2001).

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